MAGNESIUM DEPENDENCE OF ENDOTOXIN-INDUCED DEGRANULATION OF *LIMULUS* AMEBOCYTES

GAIL W. CANNON, MASAKAZU TSUCHIYA*, DAN RITTSCHOF, AND JOSEPH BONAVENTURA

*Marine Biomedical Center, Duke University Marine Laboratory, Pivers Island, Beaufort, North Carolina 28516, and *Wako Pure Chemical Industries, Ltd., Osaka Research Laboratory, Hyogo, 661 Japan*

ABSTRACT

Amebocytes are blood cells that function in the defense system of the horseshoe crab *Limulus polyphemus.* When they are withdrawn from the animal, they flatten and degranulate within hours. We have found, however, that when a small amount of blood is drawn into a syringe containing a large amount of 3% NaCl, free of divalant cations, and further diluted (to a final concentration of approximately 1:500) in 3% NaCl into tissue-culture-treated plates, amebocytes do not flatten or degranulate, even in the presence of endotoxin. This dilution technique was used to determine direct effects of Ca^{++} and Mg^{++} in the flattening and degranulation of amebocytes.

Ca^{++} or Mg^{++} added at normal hemolymph concentration to 3% NaCl caused rapid flattening. Degranulation did not occur for several days. In the presence of nanogram quantities of endotoxin, cells in *Ca^{++} fortified saline remained granulated,* while those in *Mg^{++} fortified saline completely degranulated* within several hours. Experiments with EGTA and EDTA confirmed that Ca^{++} is not essential for degranulation of amebocytes in the presence of serum. Although Ca^{++} and Mg^{++} are both influential in the *in vitro* flattening of *Limulus* amebocytes, Mg^{++} is essential for the process of degranulation.

INTRODUCTION

Granular amebocytes are the major blood cells of *Limulus polyphemus* and play an important role in the defense system. In the presence of endotoxin, factors of the extracellular gelation system are released from amebocytes. The resultant extracellular gel presumably immobilizes bacteria (Bang, 1956; Levin and Bang, 1964, 1968; Levin, 1976; Armstrong and Rickles, 1982). In the absence of endotoxin, amebocytes in culture are motile (Armstrong, 1977, 1979, 1980) and are able to phagocytose (Armstrong and Levin, 1978). In the absence of anti-aggregating chemicals such as *N*-ethylmaleimide (Solem, 1970), EDTA (Kenney *et al.,* 1972; Armstrong, 1980) propranolol (Murer *et al.,* 1975), and methylxanthine derivatives (theophilline, caffeine, theobromine) (Kobayachi and Yamamoto, 1974), amebocytes *in vitro* flatten and degranulate (Armstrong and Levin, 1978; Armstrong 1977, 1979, 1980). In order to understand better the activation of amebocytes, a modified dilution technique that prevents flattening and degranulation of amebocytes *in vitro* without the necessity for the addition of anti-aggregating chemicals was developed. Using this technique, we observed the independent effects of Mg^{++} and Ca^{++} on flattening and endotoxin-induced degranulation of amebocytes. We found that, although both Mg^{++} and Ca^{++}

caused flattening of amebocytes, only Mg^{++} was essential for endotoxin-induced degranulation.

MATERIALS AND METHODS

Animals

Limulus polyphemus were obtained throughout the year by trawl from high salinity waters (ranging near 1000 kOs) from 10°C to 29°C within three miles of the Beaufort Inlet on the eastern shore of North Carolina. The animals were maintained in outdoor, tidal-exchanged, natural-bottomed, flow-through pens, (25 feet by 25 feet, containing a maximum of 200 animals at any given time), where they were able to feed at will. No exogenous food was added. One to three days before experimentation, the animals were moved to indoor tanks of flowing seawater and maintained unfed until use.

Reagents

Water used in experiments was sterile for injection (Abbott Laboratories, North Chicago, Illinois). Endotoxin was *Escherichia coli* UKT-B lipopolysaccharide (WAKO Pure Chemical Industries, Ltd., Osaka, Japan). Potency of the endotoxin was confirmed with U.S.P. Reference Standard Endotoxin EC-5 by the Limulus Amebocyte Lysate test. The potency of this preparation was 25 EU/ng. One thousand EU of this preparation contained less than 0.005 μg of Mg^{++} and less than 0.02 μg of Ca^{++} as measured by atomic absorption. Sterile Trypan Blue (Gibco, Grand Island, Maine) was made isotonic by addition of sodium chloride. Other chemicals were reagent grade. Inorganic chemicals were rendered pyrogen-free by baking at 180°C–210°C for 4 h.

Equipment

Sterile Linbro tissue-culture-treated, multi-well plates, (Cat. No. 76-033-05), sterile Linbro non-tissue-culture-treated, multi-well plates (Cat. No. 76-258-05, Flow Laboratories, McLean, Virginia), plastic, 60×15 mm non-tissue-culture-treated Petri dishes (Falcon #1007, Oxnard, California), 16 gauge, 25.4 mm needles (#5197), and 60 cc Plastipak syringes (#5663) (Becton-Dickenson, Rutherford, New Jersey) were used. Glassware, spatulas, and forceps were rendered pyrogen-free by baking at temperatures of 180°C–210°C for 4 h. Differential interference contrast, phase, and brightfield photomicrography was employed; results were recorded on Kodak Panatomic-X 32 film (Eastman Kodak, Rochester, New York).

Bleeding animals

To obtain amebocytes, we cleansed the flexure of the animals' prosoma and opisthosoma with 70% ethanol and withdrew 1 to 2 ml of hemolymph by cardiac puncture with sterile 16 g needles attached to sterile 60 cc syringes containing 49 ml of pyrogen-free 3% NaCl.

Preparation of serum

Bulk hemolymph for serum preparation, obtained by cardiac puncture with a 13 gauge needle, was allowed to clot overnight at 4°C and was centrifuged at 2000 rpm

for 1 h to remove clotted amebocytes and cellular debris. Serum was stored until use at 4°C.

Preparation of amebocytes

Amebocytes were prepared for tissue culture experimentation as follows. Immediately after bleeding, approximately 50 to 100 μl hemolymph, diluted 1:50 with 3% NaCl, were added dropwise to tissue-culture-treated, multi-well plates containing an additional ml of 3% NaCl. Final cell concentration, determined by direct cell count with a hemocytometer, was approximately 4 to 5 $\times$ 10^4 per ml. Cells settled undisturbed for at least 1 h before experimentation.

Amebocytes were prepared for photomicrography by placing pyrogen-free coverslips in plastic Petri dishes. Cells settled onto the coverslips, which then could be removed for more effective photomicrography.

Surface effects and viability study

To determine the effects of various surfaces on amebocytes, cultures in 3% saline were tested on tissue-culture-treated, multi-well plates, non-tissue-culture-treated plastic Petri dishes, glass Petri dishes, and siliconized-glass Petri dishes. Trypan blue was used as a viability stain (Merchant *et al.*, 1964). Cells were placed in 3% NaCl into all the wells of a tissue-culture-treated, multi-well plate. Cells were kept unfed at ambient temperature and conditions. One well of cells per day was tested for viability.

Ca^{++} and Mg^{++} studies

Amebocytes were placed into multi-well, tissue-culture-treated plates as above. After the cells had settled, supernatant was removed with a sterile pipet and was replaced with isotonic saline containing calcium chloride at 0.24 mM to 15.0 mM or magnesium sulfate at 3.30 mM to 75 mM. Endotoxin was added at final concentrations of 10–2500 EU/ml to cells at all of the concentrations of Mg^{++} and Ca^{++} studied. Within 24 h of addition of Mg^{++} and Ca^{++}, coverslips with experimental cells were removed from dishes and placed uncovered on slides. Results were recorded by photomicrography with differential interference contrast optics.

EDTA and EGTA studies

Amebocytes were placed into tissue-culture-treated, multi-well plates. EDTA at 20 mM and 40 mM or EGTA at 5 mM and 10 mM was added to the serum. NaCl was removed from prepared cells and replaced with serum alone or serum containing either EDTA or EGTA at the above concentrations. Results were recorded within 24 h by photomicrography.

RESULTS

Development of dilution technique

Substrates were tested for their effects on amebocyte morphology. Amebocytes attached to tissue-culture-treated, multi-well plates but did not flatten or degranulate
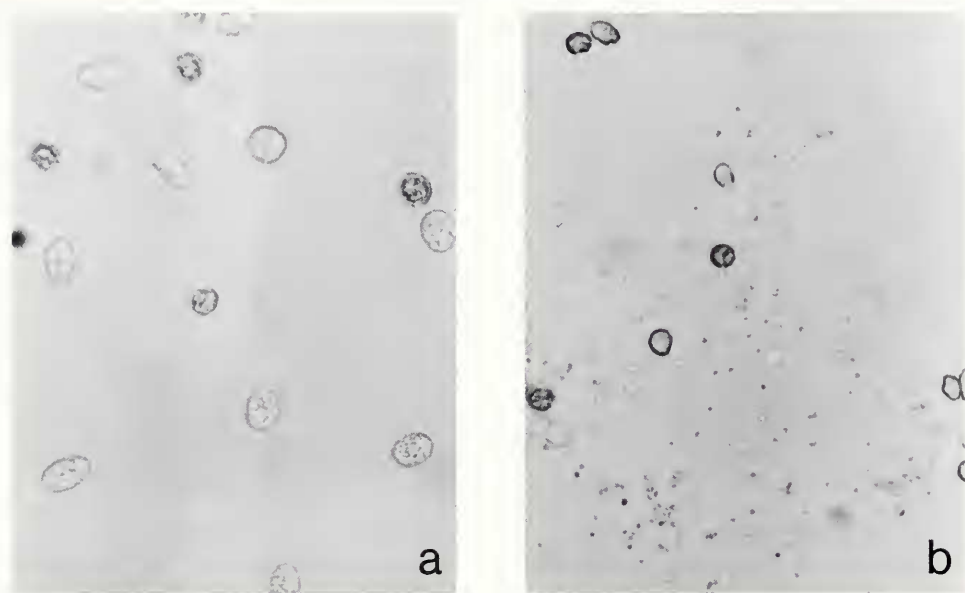
FIGURE 1.   Effects of surfaces on *Limulus* amebocytes cultured for 1 h in 3% NaCl. (a) Sterile Linbro tissue-culture-treated, multi-well plates; cells are ovoid and granulated. (b) Sterile Linbro non-tissue-culture-treated, multi-well plates; intact granules released from lysed cells. Brightfield photomicrographs, ×200.

until death (Fig. 1a). Trypan blue exclusion tests indicated that these cells remained alive for approximately one week. In non-tissue-culture-treated plastic dishes, many cells disintegrated upon contact with the surface and others attached but contracted and became generally smaller and more rounded. Granules from cells that disintegrated remained intact (Fig. 1b). On siliconized glass surfaces, amebocytes attached and contracted with some disintegration similar to non-tissue-culture-treated plastic plates. Cells on non-siliconized glass Petri dishes attached and remained ovoid with some slight contraction and rounding similar to those in tissue-culture-treated, multi-well plates. Tissue-culture-treated, multi-well plates were, therefore, used experimentally because they affected cellular morphology least.

Seasonal variation was observed in the reactivity of amebocytes and their responses to surfaces. During summer months, amebocytes appeared to respond to stimuli (serum or Mg^{++}) more quickly and looked "healthier" (more regularly shaped) than did amebocytes obtained during winter months. This phenomenon did not alter the results reported.

Experiments

Amebocytes cultured in 3% NaCl remained ovoid and granulated. Amebocytes cultured in 3% NaCl and exposed to 50 mM Mg^{++} (Fig. 2a) flattened completely and remained granulated for 24 h. The process of flattening is begun in the presence of 6.25 mM Mg^{++} and is complete at concentrations of 25 mM Mg^{++} and above. A similar reaction was observed with 10 mM Ca^{++}. The process of flattening is begun in the presence of 0.5 mM Ca^{++} and is complete at concentrations of 10 mM Ca^{++}
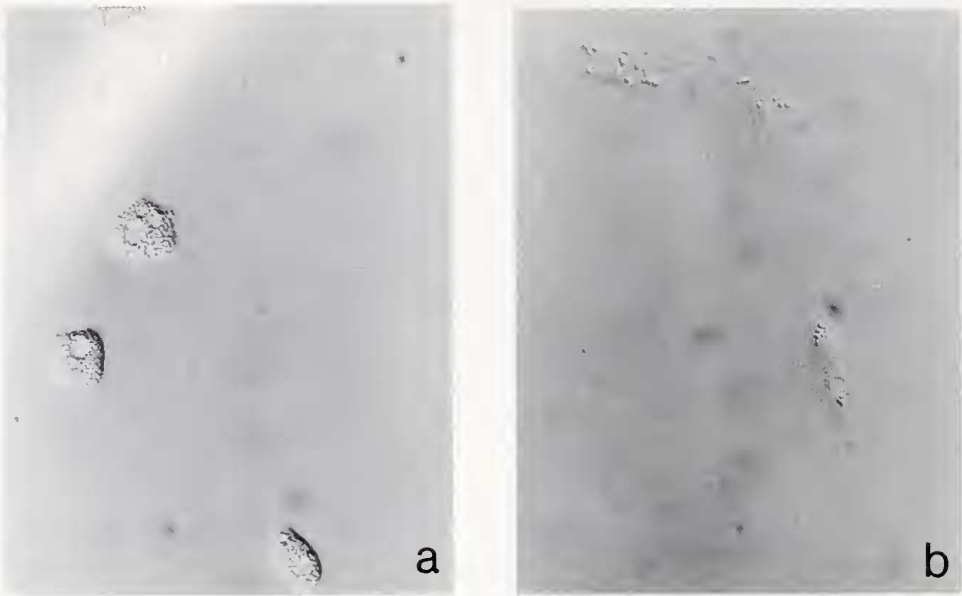
FIGURE 2.   Effects of endotoxin on *Limulus* amebocytes cultured for 6 h in 3% NaCl containing 50 mM Mg^{++} on glass coverslips. (a) 3% NaCl containing 50 mM Mg^{++} without endotoxin; cells are flat and granulated. (b) 3% NaCl containing 50 mM Mg^{++} in the presence of 125 EU/ml endotoxin; cells are flat and degranulated. Differential interference contrast photomicrographs, ×400.

and above. Various transitional stages of flattening are seen between 6.25 and 25 mM Mg^{++} and between 0.5 mM and 10 mM Ca^{++}.

Endotoxin at 2500 EU/ml added to amebocytes cultured in 3% NaCl did not cause degranulation. Amebocytes remained ovoid and granulated. When 125 EU/ml endotoxin were added to amebocytes in the presence of 50 mM Mg^{++}, degranulation was complete within 24 h (Fig. 2b). Mg^{++}-facilitated degranulation as a function of endotoxin concentration is shown in Table I. The effects of varying concentrations of Mg^{++} in the presence of constant amounts of endotoxin are shown in Table II. The amount of endotoxin necessary to produce degranulation was dependent on Mg^{++} concentration. A minimum of 25 mM Mg^{++} was required for the degranulation of amebocytes in the presence of 2500 EU/ml of endotoxin. When endotoxin in concentrations ranging from 10 to 2500 EU/ml was added to amebocytes in the presence of 0.1 to 15 mM Ca^{++}, the amebocytes remained granulated.

TABLE I

Mg^{++} supported degranulation of amebocytes in the presence of endotoxin

Mg^{++} (mM)	Endotoxin (EU/ml)							
	0.0	15.6	31.2	62.5	125	250	1000	2500
0	+	+	+	+	+	+	+	+
50	+	+	+	±	−	−	−	−

+ = granulated, ± = partially granulated, − = degranulated.

TABLE II

Endotoxin-induced degranulation of amebocytes in the presence of Mg⁺⁺

Endotoxin (EU/ml)	Mg⁺⁺ (mM)						
	0.0	0.5	3.1	6.2	12.5	25.0	50.0
0	+	+	+	+	+	+	+
1000	+	+	+	+	±	±	−
2500	+	+	+	±	±	−	−

+ = granulated, ± = partially granulated, − = degranulated.

Amebocytes cultured in 10 to 100% *Limulus* serum flattened and also degranulated within 24 h. EDTA at 20 mM and 40 mM in hemolymph serum prevented amebocytes from flattening and degranulating. However, amebocytes exposed to 5 mM and 10 mM EGTA-supplemented serum flattened and degranulated within 24 h.

DISCUSSION

Amebocytes, when placed on negatively charged surfaces, (glass and tissue-culture-treated, multi-well plates), in 3% NaCl, attached but retained the *in vivo* characteristics of ovoid shape and granule retention for one week (Fig. 1a). The phenomenon of amebocytes disentegrating with intact granule release when placed on hydrophobic surfaces (siliconized glass and non-tissue-culture-treated, plastic dishes) (Fig. 1b) may be a result of interaction between these surfaces and cell membranes. The technique of obtaining amebocytes with few morphological changes in the absence of anti-aggregating chemicals will be useful for future studies on amebocyte activation.

Partial flattening and spontaneous degranulation of amebocytes on artificial surfaces have been reported (Armstrong, 1980). Divalent cations and unknown hemolymph components have been considered to be causal factors in these cellular modifications (Armstrong, 1980). In this study, flattening of amebocytes cultured in 3% NaCl resulted from the addition of Mg⁺⁺, Ca⁺⁺, or hemolymph serum. Spontaneous degranulation followed the addition of hemolymph serum. To prevent or delay these reactions without anti-aggregating chemicals, immediate dilution by a factor of at least 500 was necessary. Amebocytes obtained by this method, although not in an *in vivo* state, had not begun the processes of flattening and degranulation.

Amebocytes cultured in 3% NaCl began to flatten in the presence of greater than 12.5 mM Mg⁺⁺ or 1.0 mM Ca⁺⁺. In the absence of endotoxin these amebocytes retained their granules for more than 24 h.

Endotoxin causes the gelation of *Limulus* hemolymph (Levin and Bang, 1964). Gelation follows the exocytosis of amebocyte granules, which contain all of the factors of the gelation system (Murer *et al.*, 1975; Ornberg and Reese, 1981; Armstrong and Rickles, 1982). No triggering mechanism for exocytosis has been reported. As a result of the dilution technique, endotoxin could be added in amounts as great as 2500 EU/ml with *no resultant degranulation* of amebocytes cultured in 3% NaCl. This failure to degranulate indicates that degranulation requires the presence of a factor other than endotoxin. Our studies indicate that Mg⁺⁺ is that factor. In the presence of 50 mM Mg⁺⁺, degranulation occurred rapidly among amebocytes when more than 125 EU/ml endotoxin were added (Table I). Higher concentrations of

endotoxin required less Mg^{++} for degranulation to occur (Table II). Degranulation of amebocytes did not occur in the presence of calcium even at a concentration of 2500 EU/ml endotoxin. Therefore, Mg^{++}, but not Ca^{++}, is essential for endotoxin-induced degranulation. Although magnesium-dependent exocytosis may not be unique in nature, we have found no similar reports. This presents a challenge to examine more closely the role of Mg^{++} as a trigger for biological events.

The observation that EDTA inhibited spontaneous degranultion of amebocytes in serum and EGTA did not inhibit degranulation supports the assertion that Ca^{++} *is not* necessary for the degranulation of amebocytes. EDTA chelates both Mg^{++} and Ca^{++} while EGTA chelates Ca^{++} but does not chelate Mg^{++}. Spontaneous degranulation occurs within hours in amebocytes cultured in hemolymph (Armstrong, 1980, 1982). When cells were cultured by the dilution technique, complete degranulation was delayed. Eventual degranulation may be due to residual amounts of Mg^{++} and other serum factors added as part of the original innoculum.

Aggregation of amebocytes and flattening and degranulation of amebocytes are separable processes. There is a serum factor that causes aggregation even in the presence of EDTA (Kenney *et al.,* 1972). Our observations indicate that EDTA inhibits both flattening and degranulation even in the presence of hemolymph that contains this aggregating factor.

Limulus hemolymph contains 46 mM Mg^{++} and 10 mM Ca^{++} (Prosser, 1973), sufficient amounts to cause flattening of amebocytes in culture. However, amebocytes not only flatten but also degranulate in the presence of serum even in the absence of endotoxin; and since Mg^{++} and Ca^{++} do not cause degranulation, the presence of a "degranulation-promoting" factor in hemolymph serum is indicated. It appears that spontaneous degranulation, described by Armstrong (1982), is caused by this "degranulation-promoting" factor present in hemolymph serum. This factor may be released from tissues or amebocytes when *Limulus* has been injured or irritated.

Our observations that endotoxin in the presence of Mg^{++} causes degranulation and that degranulation occurs in the presence of serum in the *absence* of endotoxin suggest two possible series of events. The *degranulation-promoting* factor may have binding sites analogous to those of the lipopolysaccharide molecule and, therefore, may activate amebocytes to degranulate through the same pathway or mechanism. Alternatively, there may be two separate mechanisms that initiate amebocyte degranulation. One pathway may involve the reaction of Mg^{++} with lipopolysacharride leading to degranulation since the presence of Mg^{++} appears to be necessary for degranulation to occur. A second pathway may involve separate reactions, involving the *degranulation-promoting* factor, that may lead to a link with the lipopolysaccharide/Mg^{++} pathway. Further research is needed to test these hypotheses. Another area of possible future research involves the question of whether Mg^{++} acts intracellularly or extracellularly.

LITERATURE CITED

ARMSTRONG, P. B. 1977. Interaction of the motile blood cells of the horseshoe crab, *Limulus.* Studies on contact pseudopodial activity and cellular overlapping *in vitro. Exp. Cell Res.* **107**: 127–138.
ARMSTRONG, P. B., AND J. LEVIN. 1978. *In vitro* phagocytosis by *Limulus* blood cells. *J. Invert. Pathol.* **34**: 145–151.

ARMSTRONG, P. B. 1979. Motility of the *Limulus* blood cell. *J. Cell Sci.* **37**: 169–180.

ARMSTRONG, P. B. 1980. Adhesion and spreading of *Limulus* blood cells on artificial surfaces. *J. Cell Sci.* **44**: 243–262.

ARMSTRONG, P. B., AND F. R. RICKLES. 1982. Endotoxin-induced degranulation of the *Limulus* amebocyte. *Exp. Cell Res.* **140**: 15–24.

BANG, F. B. 1956. A bacterial disease of *Limulus polyphemus. Bull. Johns Hopkins Hosp.* **98**: 325–351.

KENNEDY, D. M., F. A. BELAMARICH, AND D. SHEPRO. 1972. Aggregation of horseshoe crab (*Limulus polyphemus*) amebocytes and reversible inhibition of aggregation by EDTA. *Biol. Bull.* **143**: 548–567.

KOBAYASHI, M., AND M. YAMAMOTO. 1974. Preparation of pre-gel from the amebocytes of Japanese horseshoe crab (*Tachypleus tridentatus*) with the anti-aggregating effect of methylxanthines. II. *Yakugaku Zasshi* **94**(3): 198–303.

LEVIN, J., AND F. B. BANG. 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* **115**: 265–274.

LEVIN, J., AND F. B. BANG. 1968. Clottable protein in *Limulus:* its localization and kinetics of coagulation by endotoxin. *Thromb. Diath. Haemorrh.* **19**: 186–197.

LEVIN, J. 1976. Blood coagulation in the horseshoe crab (*Limulus polyphemus*): a model for mammalian coagulation and hemostasis. Pp. 87–102 in *Animal Model of Thrombosis and Hemorrhagic Diseases.* DHEW Publication No. (NIH) 76-982, Washington, DC.

MERCHANT, D. J., R. H. KAHN, AND W. H. MURPHEY, JR. 1964. *Handbook of Cell and Organ Culture.* Pp. 155–210. Burgess Publishing Co., Minneapolis.

MURER, E. H., J. LEVIN, AND R. HOLME. 1975. Isolation and studies of the granules of the amebocytes of *Limulus polyphemus,* the horseshoe crab. *J. Cell Physiol.* **86**: 533–542.

ORNBERG, R. L., AND T. S. REESE. 1981. Beginning of exocytosis captured by rapid-freezing of *Limulus* amebocytes. *J. Cell Biol.* **90**: 40–54.

PROSSER, C. L. 1973. *Comparative Animal Physiology,* 3rd ed. p. 85. Saunders, Philadelphia.

SOLEM, N. O. 1970. Some characteristics of the clottable protein of *Limulus polyphemus* blood cells. *Thromb. Diath. Haemorrh.* **23**: 170.